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Cytochemical and Histological Details of Muscle Fibers in the Southern Smelt *Retropinna retropinna* (Pisces; Galaxioidei)

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ABSTRACT—Cytochemical and histological details are presented on the localization of actomyosin adenosine triphosphatase (am-ATPase), succinic dehydrogenase, lipid, and glycogen in the body musculature of the Southern smelt *Retropinna retropinna*. The results clearly show that in addition to the red and white muscle fibers a variety of pink fibers are present, which can be further classified according to fiber diameter, staining characteristics, and location. Using acid and alkaline preincubation with myofibrillar am-ATPase stain, both large and small diameter pink fibers were distinguishable from red as well as white fibers. It is suggested that a transition between aerobic and anaerobic metabolisms exists in intermediate zone fibers of which those with small diameters are likely to possess aerobic and those of large diameters a more anaerobic capacity. The difference in metabolism could be related to fiber diameter and the associated constraints of large diffusion distances on oxygen availability.

INTRODUCTION

Early histochemical studies on the localization and activities of oxydative enzymes in vertebrate muscles [19] have shown that muscle fibers can be classified into three types. These three types are traditionally referred to as red, white, and pink in fishes. However, the development of the technique for the demonstration of myofibrillar adenosine triphosphatase (ATPase) for mammalian muscle [8] and its modification for fish muscle [12] has allowed the employment of an alternative classification based on myofibrillar ATPase activity. According to Johnston *et al.* [13] and numerous other investigators [3, 6, 9, 14, 18, 22, 24, 25] fiber types within the intermediate (=pink) group can be further subdivided.

They display a variety of staining activities, but are characterized by their relatively small diameter and a gradation in staining intensity for ATPase after acid and alkaline preincubation [6, 25]. As part of a study on red/white muscle distribution and fiber growth dynamics in different populations of the Southern smelt *Retropinna retropinna,* we decided to commence with a histochemical survey of fiber types present in sections of the fish corresponding to 70% fork length, i.e. the region located near the point of maximum flexure [17].

MATERIALS AND METHODS

Specimens of the Southern smelt, *Retropinna retropinna,* were caught during the day and late evenings at two locations along the Waikato River (New Zealand) or in Lake Rotomanuka located 10 km to the south of Hamilton (New Zealand). The fish were kept in a large aquarium in a 15°C constant temperature room.

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All histological studies were carried out using frozen material. The smelt were killed by either decapitation or pithing and their fork length, total weight, and sex were recorded. To be able to compare results, a consistent sampling regime was adopted, which involved sampling from a point corresponding to 70% of the fork length.

Blocks of fish myotomal muscle were immersed in isopentane cooled to near its freezing point by liquid nitrogen, as soon as possible after death. They were then mounted on cryostat chucks that had been cooled in liquid nitrogen using O.C.T.-compound, an embedding medium for frozen tissue specimens. The samples were then placed in a cryostat set at −20°C to equilibrate for 1 hr. The specimen blocks were sectioned at a thickness of 10–20 μm and the sections were mounted on glass slides and allowed to dry for 30 min.

Sections were stained for glycogen using the periodic acid Schiff's method [7] and for lipid using Sudan Black B. Sections were stained for succinic dehydrogenase activity using the nitro-blue tetrazolium method of Dubowitz and Brooke [7] or the method of Pearse [21]. The myofibrillar adenosine triphosphatase (ATPase) activity was demonstrated by using the method of Guth and Samaha [8] as modified by Johnston *et al.* [12]. Sections were preincubated, without prior fixation in the alkaline (pH 10.4) solution at room temperature (21°C). The time of preincubation was adjusted until a differential pattern of staining was obtained without total inactivation of all fibers (30–300 sec). Some sections were also preincubated in the acidic (pH 4.35, 30–90 sec) preincubation solution at room temperature (21°C) for comparative purposes [18]. The preincubated sections were then incubated for 20 min at 37°C.

As a control, some sections were incubated with 3.5 mM sodium azide in the incubation solution to inhibit Ca^{2+}-activated mitochondrial ATPase [13]. Freshly frozen muscle tissue was used as muscle tissue stored in liquid nitrogen for prolonged periods before sectioning and incubation, was found to have a radically changed pattern of ATPase activity. White muscle ATPase activity decreased and red muscle ATPase increased, which is similar to that found by Korneliussen *et al.* [14]. Control sections in the PAS-technique for glycogen were treated in a 0.5% alpha-amylase solution for 10–15 min before staining [15].

In the Sudan Black B technique for the presence of lipid, counterstaining in filtered haematoxylin was omitted as good results were obtained without it. Some sections were immersed in acetone for 30 min prior to staining to act as a control [18]. To demonstrate the activity of the mitochondrial enzyme succinic dehydrogenase, the technique of Dubowitz and Brooke [7] was used. The results from this staining technique were then confirmed using the method of Pearse [21]. As a control the nitro-blue tetrazolium salt was omitted in some incubations.

RESULTS

The results obtained from the histological localization of actomyosin adenosine triphosphatase (am-ATPase), succinic dehydrogenase (SDH), lipid, and glycogen are presented in Tables 1, 2, and 3. The results clearly show that in addition to the red and white fibers, a variety of pink muscle fibers are present.

Table 1. Staining intensities of different fiber types in the myotomal musculature of the Southern smelt *Retropinna retropinna*

Fibre types	Staining techinique for:		
	Lipid	Glycogen	SDH
White	0	0	0
Ping large	0	+	+
Pink small	0	+, ++	++
Red	++	+++, 0#	+++
S.D.F.	+, 0	+, 0	+, 0

# red fibers of Seitenlinie and the elongate red fibers adjacent to the perimysium.
0 =Background stain
+ =Lightly stained
++ =Moderately stained
+++=Heavily stained
S.D.F =Small Diameter Fiber

Table 3. Effect of different preincubation times on the myofibrillar ATPase in the Southern smelt *Retropinna retropinna*

Fibre type	Preincubation time at pH 10.4		
	0 sec	30 sec	5 min
White	+++	++	0
Pink large	+++	+++	0
Pink small	+++	++++, +++, 0	+
Red	+++	+#	+#
S.D.F.	++, +	+	0

=Staining probably due to mitochondrial ATPase activity

The comparatively small diameter red muscle fibers were located in a superficial wedge-shaped region at the periphery of the horizontal septum adjacent to the lateral line, but they were also present in a superficial sheet 1–2 fibers thick, located between the dermis and the main mass of white fibers. The fibers of the *musculus lateralis superficiales* displayed similar staining characteristics, except for a monolayer of elongate fibers adjacent to the perimysium, which appeared to form a continuum of the "Seitenlinie" red fibers. The latter displayed staining activities that differed from the red fibers of the *lateralis superficiales* in respect to glycogen and acid-stable myofibrillar ATPase.

Table 2. Staining activities of myofibrillar ATPase under different preincubation conditions

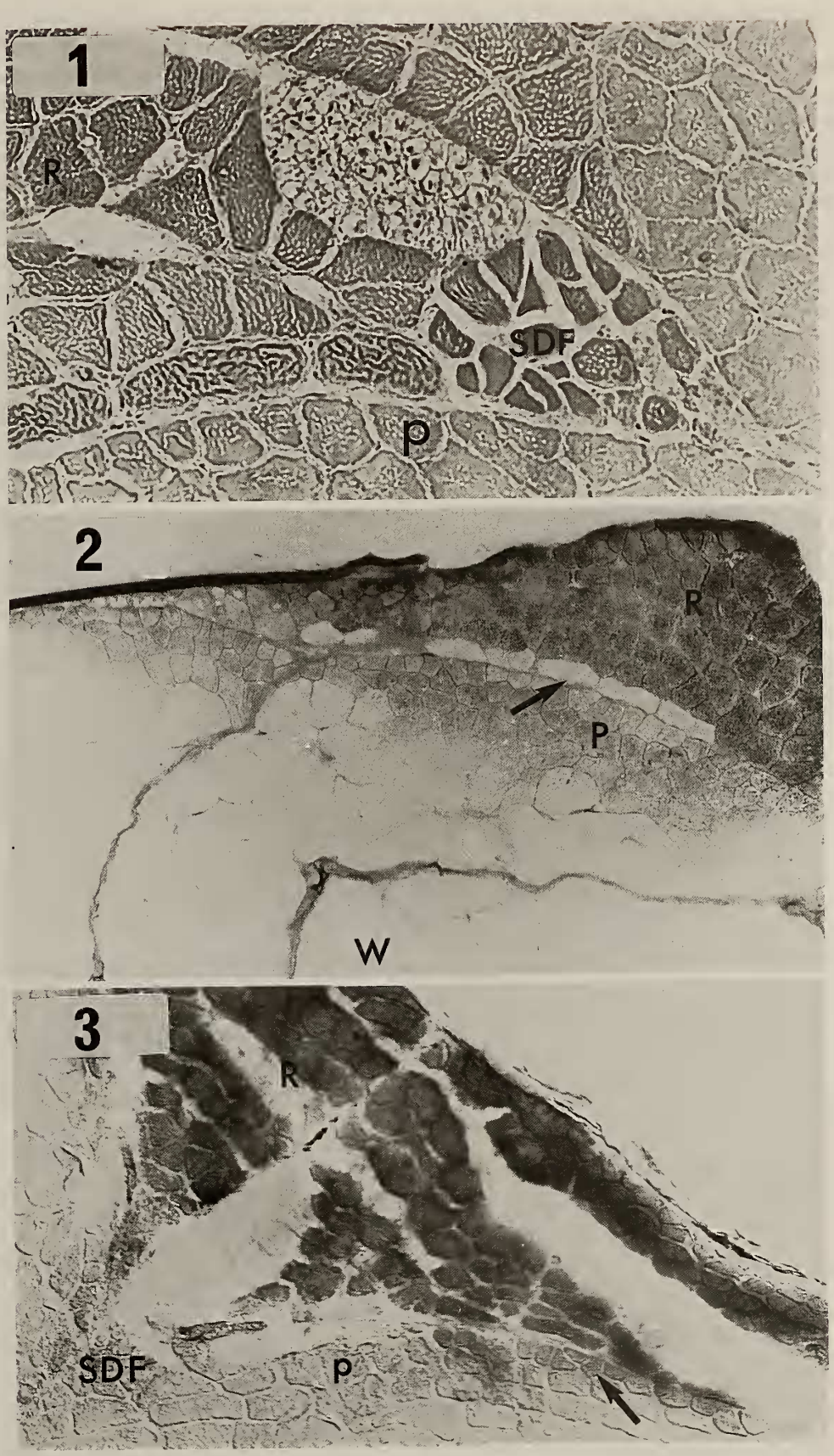
Fiber types	Myofibrillar ATPase preincubation			
	none	Alkaline pH 10.4	Acid pH 4.35	Na Azide
White	+++	++	0	++
Pink large	+++	+++	+	++
Pink small	+++	++++, +++, 0	++	+++
Red	+++	+#	++++*, +++	0
S.D.F.	++, +	+	++	0?

? =Unable to fully determine
=Staining probably due to mitochondrial ATPase activity
* =Elongated red fibers located next to the perimysium and red fibers of the seitenlinie

Fig. 1. Distribution of lipid in the lateral musculature of the smelt using Sudan Black B stain for lipid. Note intense staining in the red fibers (R) and the more intensely stained Seitenlinie fibers. Also shown is the variety of staining intensities displayed by the small diameter fibers (SDH) and the limited background staining displayed by the pink fibers (P). Magnification: 400×.

Fig. 2. Localization of glycogen within the lateral musculature of the smelt using the PAS-stain. Note gradation in staining from intense in the red fibers (R) through intermediate levels in the pink fibers (P) to no staining in the white fibers (W). Also note the lack of staining in the Seitenlinie fibers (arrow). Magnification: 125×.

Fig. 3. Distribution of mitochondrial enzyme succinic dehydrogenase in myotomal musculature of the smelt using nitro-blue tetrazolium stain for SDH. Note the gradation of staining intensities with red fibers (R), staining most small pink fibers (arrow) and large pink fibers (P) displaying intermediate staining and white fibers (W) no activity at all. Also note the small diameter fibers (SDH) show very little activity. Magnification: 125×.


1
R
SDF
P
2
R
P
W
3
R
SDF
P

The *musculus lateralis profundus,* which is composed entirely of white muscle fibers, comprises the bulk of the musculature present in the myotome. These fibers which possessed a large mean diameter, displayed a homogeneous, non-mosaic appearance with respect to staining intensities as reported for other teleosts [18, 25]. The intermediate pink fibers were categorized as small diameter pink fibers. The former were situated adjacent to the perimysium in a layer one to three fibers thick and were of a smaller mean diameter than the red fibers, displaying a variety of staining intensities for SDH and myofibrillar ATPase. The other form of intermediate muscle fibers were located between the small diameter pink fibers and the white fibers. They were classified as large diameter pink fibers according to Patterson *et al.* [20] and were distinguished by (a) their homogeneous staining intensities for all staining techniques used, and (b) an intermediate range of fiber diameters between that of red and white fiber types.

One other fiber type was sometimes observed to be located near the junction of the perimysium and additional myosepta at the point of the wedge-shaped *musculus lateralis superficialis.* When not replaced by connective tissue, these fibers were differentiated from the red muscle fibers by their extremely small mean diameter and their low staining intensities. These fibers were classified as small diameter fibers as they displayed similar properties to the small diameter fibers described by Johnston [10] and Davison [5].

Sudan Black B Technique for lipid

The staining of red muscle fibers for lipid using the Sudan Black B was relatively intense and could be used to distinguish them from white and intermediate pink fibers (Fig. 1). The white and pink fibers developed no significant staining for lipid, except for their cellular boundaries. This was probably due to lipoprotein complexes of the cell membranes or extracellular lipid. Within the intermediate fiber types no differentiation was observed between the small diameter and large diameter pink fibers. The small diameter fibers, when present, also developed no significant staining activity.

PAS stain for glycogen (Fig. 2)

Red fibers displayed intense staining activity for glycogen. A heterogeneous effect was observed in most preparations with a small number of fibers, consisting of elongate fibres adjacent to the perimysium, displaying little or no staining activity for glycogen. The red fibres of the *Seitenlinie* displayed a variety of staining intensities, from moderate to no staining at all. The variety of staining patterns apparent in the red fibers was dependent on the immediate past activity of the fish and may possibly display some hierarchy of recruitment or function resulting in glycogen depletion [23].

Succinic dehydrogenase stain

The staining pattern for SDH was similar to glycogen, that is the red fibers staining intensely and the white fibers showing no staining activity at all. The two categories of intermediate fibers exhibited differential staining using this technique with the large diameter pink fibers displaying moderate staining activity localized primarily at the cell boundary. The small diameter pink fibers developed a moderate staining intensity which lay between that of the red fibers and that of the large diameter pink fibers (Fig. 3). The small diameter fibers again displayed little or no staining activity (Fig. 3).

Myofibrillar adenosine triphosphatase

The pattern of staining for myofibrillar ATPase was found to be dependent on both the pH and duration of preincubation (Tables 2 and 3). All the muscle fiber types present in the myotome stained heavily when incubated for myofibrillar ATPase without preincubation. A differential pattern of staining was obtained by preincubating sections at pH 10.4 for a period of 30 sec at 21°C (Figs. 4, 5, and 7). Longer periods of preincubation were found to cause inactivation of the ATPase in all fiber types, though some staining of pink fibers was still evident.

The reportedly high alkali-stable myofibrillar ATPase activity present in white fibers displayed a variable intensity of staining for different alkaline pH values, and was completely inactivated in a relatively short period of time (Table 3). These fibers displayed no activity when incubated for acid-stable ATPase using acidic preincubation (Fig. 6).

The staining pattern of white fibers was also altered if the specimens were stored in liquid nitrogen for more than several days, resulting in inactivation of the enzyme (Fig. 8).

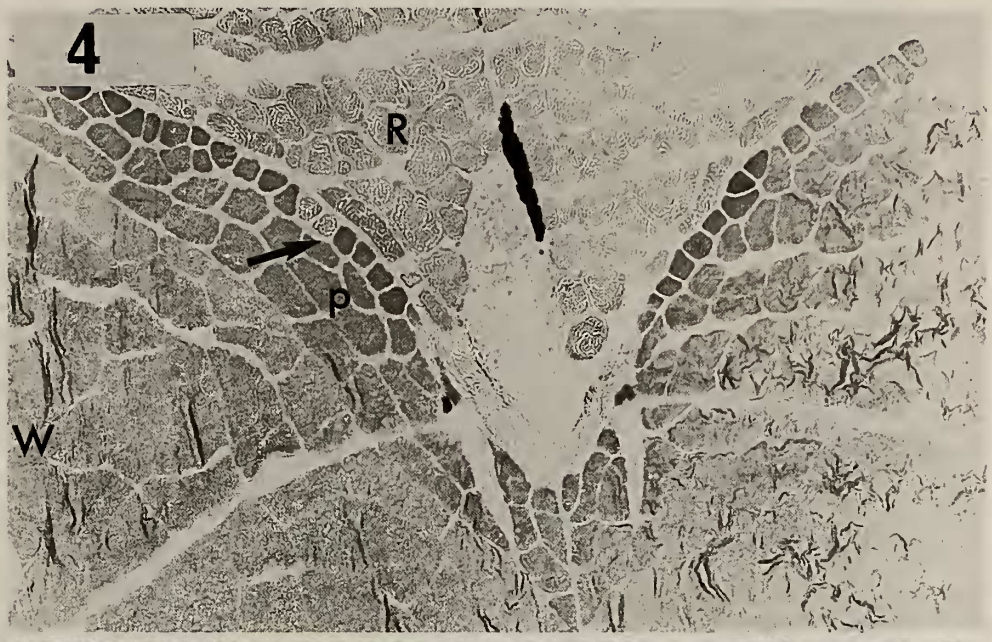
The alkali-labile ATPase present in the red fibers of the *musculus lateralis superficiales* also demonstrated a variation in staining over the whole range of pH values tested. Alkaline preincubation, which is generally considered to inactivate red myofibrillar ATPase actually resulted in staining of these fibers. A staining pattern was, thus, produced showing alkali-stable ATPase activity in white, red, and intermediate

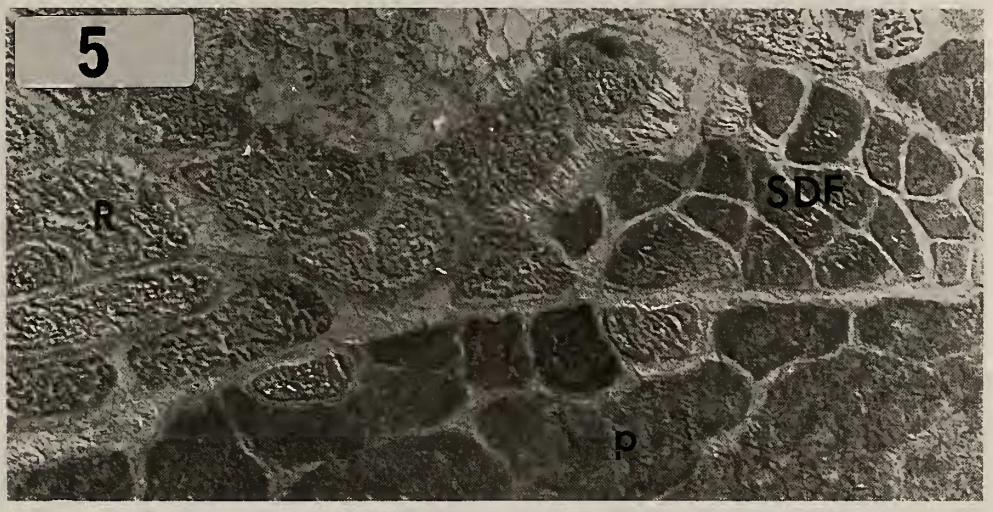
Fig. 4. Demonstration of alkali-stable myofibrillar ATPase in the myotomal musculature. Note the low staining intensity of the red fibers (R), moderate staining of white fibers (W), the more intense staining of the large diameter pink fibers (P), and the variety of staining patterns exhibited by the small diameter pink fibers (arrow). Magnification: 125×.

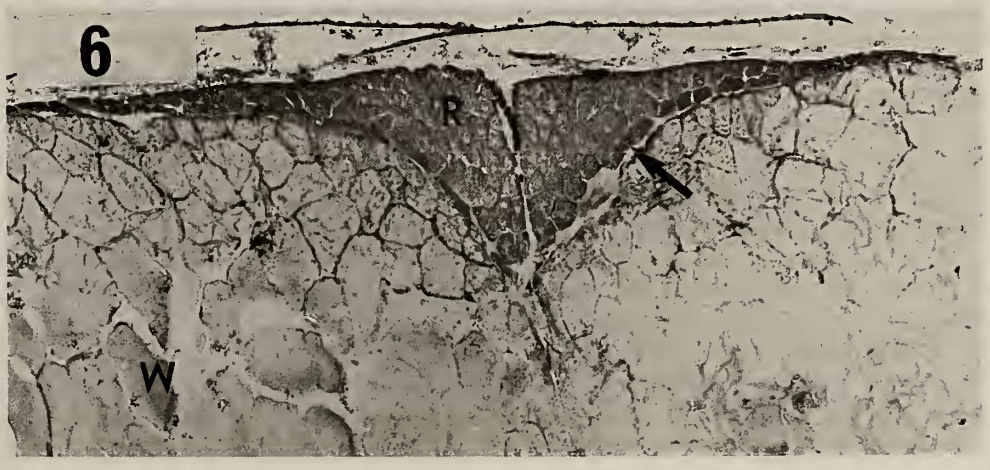
Fig. 5. Localization of alkali-stable myofibrilla ATPase in the myotomal musculature. Note the staining of the red fibers (R) due to Ca^{2+}-activated mitochondrial ATPase, which results in a granular appearance. Also of importance is the variety of staining intensities exhibited by the small diameter pink fibers (P) and small diameter fibers (SDH). Magnification: 400×.

Fig. 6. Demonstration of acid-stable myofibrillar ATPase in the myotomal musculature. Note that the red (R) and some pink small diameter fibers were the only fibers that stained. White (W) and large pink fibers show no staining activity. An important point to note is the intense staining of the Seitenlinie fibers (arrow). Magnification: 80×.

pink fibers. The alkali-stable staining activity of the fibers was determined to be due to Ca^{2+}-activated mitochondrial ATPase, as this was the only staining artefact abolished (other fibers stayed the same), if sections were incubated in the presence of 3.5 mM sodium azide, a known inhibitor of mitochondrial activity. Acid-stable myofibrillar ATPase activity developed relatively intensely in the red fibers with the Seitenlinie and elongated red fibers adjacent to the







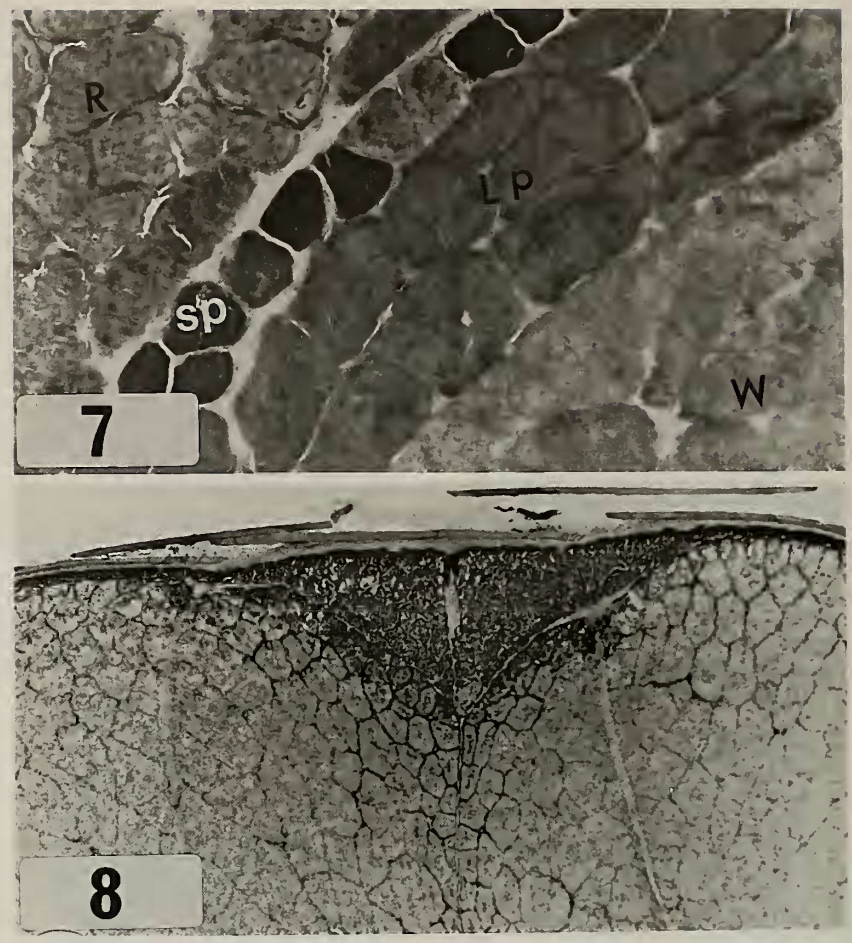


Fig. 7. Localization of alkali-stable myofibrillar ATPase in the myotomal musculature. Note differential staining of the intermediate fibers with small diameter pink fiber (SP) exhibiting a variety of staining intensities while the large pink fibers (LP) are relatively homogeneous. Also of importance is the intensity of staining shown by intermediate fibers, which is more intense than either that of the white (W) or the red (R) fibers. Magnification: 400×.

Fig. 8. Consequence of storage in liquid nitrogen for one week on alkali-stable myofibrillar ATPase activity. Note that the staining activity is greatly reduced in the white muscle with red and some pink fibers showing the only staining activity of any greater degree. Magnification: 80×.

perimysium displaying more activity (Fig. 6).

Both the large and small diameter pink fibers were distinguishable from red and white fibers using alkaline and acid preincubation (Figs. 4–7). In the histochemical demonstration of the alkali-stable ATPase, the large diameter pink fibers stained differently from the small diameter pink fibers (Figs. 4 and 7). A homogeneous staining pattern was produced in the large diameter fibers which displayed higher alkali-stable ATPase activity than the white and red fiber types (Fig. 7). The small diameter pink fibers showed a variety of staining intensities ranging from the darkest of all the fiber types to others displaying an intensity similar to that of red fibers.

In acidic preincubation, both types of pink fibers exhibited a moderate staining activity, with the small diameter fibers having stained slightly darker (Fig. 6). The staining patterns in both fibers, when viewed under high magnification, appeared granular in appearance with most of the stain localized along the periphery and in granules throughout the cell. These locations probably correspond to the positions of subsarcolemmal staining for myofibrillar ATPase with no preincubation and slight staining for alkalistable ATPase. The activity for acid-stable ATPase was marginally more intense.

DISCUSSION

In muscle, metabolic differentiation of fiber types is related to the enzymatic organization of the systems supplying energy [2]. Lipid is an important fuel particularly in species that migrate. It also seems likely that oxidation of fats as fuels is much more important in red than in white muscle as reflected by strong staining for lipid in red fibers [16]. The red fibers of the *Seitenlinie* and those elongate fibers next to the perimysium displayed similar lipid and SDH-activities to the other red fibers, but glycogen levels were not as high. Two possible explanations are that oxidation of lipids is the main source of energy for these fibers or

that during the preparation for this technique these fibers were recruited, resulting in total depletion of their glycogen stores.

The staining pattern of white muscle fibers when stained for SDH, lipid, and glycogen suggests that these fibers have only small concentrations of lipid and glycogen and low densities of mitochondria. From these results it can be concluded that white muscle fibers posses a predominantly anaerobic metabolism and lack the ability to oxidize lipids as an energy source for ATP. Therefore, utilization of glycogen in glycolysis and lactate fermentation appears to be the probable source of ATP for white fiber metabolism. Johnston and Goldspink [11], who were studying glycogen utilization in the myotomal musculature of the Crucian carp, concluded that the low levels of glycogen found in the white muscle fibers were still significant in determining the metabolism of that muscle type. They found that glycogen was utilized at levels two to three times higher in red than in white but since red muscle comprises such a small amount of the musculature, it accounts for only 15–20% of the total glycogen utilized.

The intermediate fibers displayed a differential pattern of staining from red and white fiber types. Patterson *et al.* [20] subdivided the pink fibers on the basis of size and histochemical profiles into large and small categories, which resembled the white and red fibers, respectively, except in staining for SDH where both categories stained equally lightly. In this study the two categories stained differentially for SDH, with the small diameter pink fibers staining more intensely than the large ones. Mosse and Hudson [18] reported a similar staining pattern and suggested that graded staining patterns (obtained when stained for SDH, glycogen) implied that both the large and small diameter pink fibers represented a transition zone between the red and white muscle mass. It is, therefore likely that not only an anatomical, but a functional transition between aerobic and anaerobic metabolism exists in this intermediate zone with the small diameter fibers possessing an aerobic and the large fibers having a more anaerobic capacity. This could possibly relate to fiber diameter and the constraints of large diffusion distances on oxygen availability. The source of energy for the metabolism for these fibers is one factor that contradicts the hypothesis of a gradual transition zone between red and white fibers.

No staining for lipid was evident in any of the intermediate pink fibers, but the presence of glycogen was obvious in all of them. Previous histological studies on a number of fish species [1, 6, 12, 18, 22, 24] have demonstrated that small diameter fibers are characterized by poor staining characteristics compared to other fiber types, possessing low lipid content and low SDH-activity, but differing in respect to the pH stability of their myofibrillar ATPase. The small diameter fibers present in the smelt displayed similar staining characteristics to those cited in previous studies. This pattern of staining implies a fiber with very low metabolic activity, which would correlate well with its function as a tonic fiber used in postural support as in the fishes [4].

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